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<p>(21) International Application Number: PCT/GB96/00916 (22) International Filing Date: 16 April 1996 (16.04.96) (30) Priority Data: 9508204.6 21 April 1995 (21.04.95) GB (71) Applicants (for all designated States except US): THE SPEY-WOOD LABORATORY LIMITED [GB/GB]; 14 Kensington Square, London W8 5HH (GB). MICROBIOLOGICAL RESEARCH AUTHORITY [GB/GB]; Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wiltshire SP4 0JG (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): FOSTER, Keith, Alan [GB/GB]; The Croft, Southampton Road, Whaddon, Salisbury, Wiltshire SP5 3DX (GB). DUGGAN, Michael, John [GB/GB]; 27A Moreton Place, Pimlico, London SW1V 2NL (GB). SHONE, Clifford, Charles [GB/GB]; 44 Oakwood Grove, Alderbury, Wiltshire SP5 3BN (GB). (74) Agent: DAVIES, Jonathan, Mark; Reddie & Grose, 16 Theobalds Road, London WC1X 8PL (GB).</p>	<p>(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report.</p>	
<p>(54) Title: BOTULINUM TOXIN DERIVATIVES ABLE TO MODIFY PERIPHERAL SENSORY AFFERENT FUNCTIONS (57) Abstract The invention relates to an agent specific for peripheral sensory afferents. The agent may inhibit the transmission of signals between a primary sensory afferent and a projection neuron by controlling the release of at least one neurotransmitter or neuromodulator from the primary sensory afferent. The agent may be used in or as a pharmaceutical for the treatment of pain, particularly chronic pain.</p>		

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Botulinum toxin derivatives able to modify peripheral sensory afferent functions**Technical Field**

This invention relates to a novel agent that is able to modify peripheral afferent function. The agent may inhibit neurotransmitter release from discrete
5 populations of neurons, and thereby reduce, or preferably prevent, the transmission of afferent pain signals from peripheral to central pain fibres. The agent may be used in or as a pharmaceutical for the treatment of pain, particularly chronic pain.

Background

10 The sense of touch has traditionally been regarded as one of the five classical senses, but in reality it is highly complex, transducing a number of different sensations. These sensations are detected in the periphery by a variety of specialised nerve endings and associated structures. Some of these are specific for mechanical stimuli of various sorts such as touch, pressure, vibration, and
15 the deformation of hairs or whiskers. Another class of nerves is able to detect temperatures, with different fibres being activated by heat and cold. A further population of nerve endings is not normally excited by mild stimuli, but by strong stimuli only. Sensory nerves of this category often respond to more than one stimulus, and are known as high-threshold polymodal fibres. They may be
20 used to sense potentially damaging situations or objects. The polymodal fibres also transduce chemical signals such as the "burning" sensation evoked by acid. Thus, the sense of touch can transmit a very detailed description of objects and serve to both inform and warn of events.

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The transduction of sensory signals from the periphery to sensation itself is achieved by a multi-neuronal pathway and the information processing centres of the brain. The first nerve cells of the pathway involved in the transmission of sensory stimuli are called primary sensory afferents. The cell bodies for the primary sensory afferents from the head and some of the internal organs reside in various of the ganglia associated with the cranial nerves, particularly the trigeminal nuclei and the nucleus of the solitary tract. The cell bodies for the primary sensory afferents for the remainder of the body lie in the dorsal root ganglia of the spinal column. The primary sensory afferents and their processes have been classified histologically; the cell bodies fall into two classes: A-type are large (60-120 μm in diameter) while B-type are smaller (14-30 μm) and more numerous. Similarly the processes fall into two categories: C-fibres lack the myelin sheath that A-fibres possess. A-fibres can be further sub-divided into A β -fibres, that are large diameter with well developed myelin, and A δ -fibres, that are thinner with less well developed myelin. It is generally believed that A β -fibres arise from A-type cell bodies and that A δ - and C-fibres arise from B-type cell bodies. These classifications can be further extended and subdivided by studying the selective expression of a range of molecular markers.

Functional analyses indicate that under normal circumstances A β -fibres transmit the senses of touch and moderate temperature discrimination, whereas the C-fibres are mainly equivalent to the polymodal high-threshold fibres mentioned above. The role of A δ -fibres is less clear as they seem to have a variety of responsive modes, with both high and low thresholds.

After the activation of the primary sensory afferents the next step in the transduction of sensory signals is the activation of the projection neurons, which carry the signal to higher parts of the central nervous system such as the thalamic nuclei. The cell bodies of these neurons (other than those related to the

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cranial nerves) are located in the dorsal horn of the spinal cord. This is also where the synapses between the primary afferents and the projection neurons are located. The dorsal horn is organised into a series of laminae that are stacked, with lamina I being most dorsal followed by lamina II, etc. The different
5 classes of primary afferents make synapses in different laminae. For cutaneous primary afferents, C-fibres make synapses in laminae I and II, A δ -fibres in laminae I, II, and V, and A β -fibres in laminae III, IV, and V. Deeper laminae (V-VII, X) are thought to be involved in the sensory pathways arriving from deeper tissues such as muscles and the viscera.

10 The predominant neurotransmitter at the synapses between primary afferents and projection neurons is glutamate, although importantly the C-fibres contain several neuropeptides such as substance P and calcitonin-gene related peptide (CGRP). A-fibres may also express neuropeptides such as neuropeptide Y under some circumstances.

15 The efficiency of transmission of these synapses can be altered via descending pathways and by local interneurons in the spinal cord. These modulatory neurons release a number of mediators that are either inhibitory (e.g. opioid peptides, glycine) or excitatory (e.g. nitric oxide, cholecystokinin), to provide a mechanism for enhancing or reducing awareness of sensations.

20 A category of sensation that requires such physiological modulation is pain. Pain is a sensation that can warn of injury or illness, and as such is essential in everyday life. There are times, however, when there is a need to be able to ignore it, and physiologically this is a function of, for example, the opioid peptides. Unfortunately, despite these physiological mechanisms, pain can
25 continue to be experienced during illnesses or after injuries long after its utility has passed. In these circumstances pain becomes a symptom of disease that would be better alleviated.

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Clinically, pain can be divided into three categories: (1) Acute pain, usually arising from injury or surgery that is expected to disappear when that injury is healed. (2) Chronic pain arising from malignant disease; the majority of people with metastatic cancer have moderate to severe pain and this is resolved either
5 by successful treatment of the disease or by the death of the patient. (3) Chronic pain not caused by malignant disease; this is a heterogeneous complaint, caused by a variety of illnesses, including arthritis and peripheral neuropathies, that are usually not life-threatening but which may last for decades with increasing levels of pain.

10 The physiology of pain that results from tissue damage is better understood than that which is caused by central nervous system defects. Under normal circumstances the sensations that lead to pain are first transduced by the A δ - and C-fibres that carry high threshold signals. Thus the synapses in laminae I and II are involved in the transmission of the pain signals, using glutamate and
15 the peptides released by C-fibres to produce activation of the appropriate projection neurons. There is, however, evidence that in some chronic pain states other A-fibres (including A β -fibres) can carry pain signals, and thus act as primary nociceptive afferents, for example in the hyperalgesia and allodynia associated with neuropathic pain. These changes have been associated with the
20 expression of peptides such as neuropeptide Y in A fibres. During various chronic pain conditions the synapses of the various sensory afferents with projection neurons may be modified in several ways: there may be changes in morphology leading to an increase in the number of synapses, the levels and ratios of the different peptides may change, and the sensitivity of the projection
25 neuron may change.

Given the enormity of the clinical problem presented by pain, considerable effort has been expended in finding methods for its alleviation. The most commonly used pharmaceuticals for the alleviation of pain fall into two

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categories: (1) Non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin and ibuprofen; (2) Opioids, including morphine.

NSAIDs have their main analgesic action at the periphery by inhibiting the production of prostaglandins by damaged tissues. Prostaglandins have been
5 shown to be peripheral mediators of pain and inflammation and a reduction in their concentration provides relief to patients. This is especially the case in mild arthritic disease, where inflammation is a major cause of pain. It has been suggested that prostaglandins are involved in the mediation of pain in the spinal cord and the brain; this may explain why NSAIDs have analgesic effects in
10 some pain states that do not involve inflammation or peripheral tissue damage. As prostaglandins, however, are only one of several mediators of pain NSAIDs alone are only effective in reducing some types of mild pain to acceptable levels. They are regarded as having a ceiling of activity above which increasing doses do not give increasing pain relief. Furthermore they have side effects that
15 limit their usefulness in chronic complaints. The use of NSAIDs is associated with irritation of the gastro-intestinal tract and prolonged use may lead to the development of extensive ulceration of the gut. This is particularly true in elderly patients who form the largest cohort of patients with, for example, arthritis.

20 Opioids act at the level of the spinal cord to inhibit the efficiency of neurotransmission between the primary nociceptive fibres (principally C-fibres) and the projection neurons. They achieve this by causing a prolonged hyperpolarization of both elements of these synapses. The use of opioids is effective in alleviating most types of acute pain and chronic malignant pain.
25 There are, however, a number of chronic malignant pain conditions which are partly or completely refractory to opioid analgesia, particularly those which involve nerve compression, e.g. by tumour formation. Unfortunately opioids also have unwanted systemic side-effects including: (1) depression of the

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respiratory system at the level of the respiratory centres in the brain stem; (2) the induction of constipation by a variety of effects on the smooth musculature of the gastro-intestinal tract; and (3) psychoactive effects including sedation and the induction of euphoria. These side effects occur at doses similar to those that produce analgesia and therefore limit the doses that can be given to patients.

Delivery of opioids at the spinal level can reduce the side-effect profile, but requires either frequently repeated spinal injections or fitting of a catheter, both of which carry increased risk to the patient. Fitting of a catheter requires that the patient is essentially confined to bed thus further restricting their quality of life.

The use of opioids for the treatment of some other types of chronic pain is generally ineffective or undesirable. Examples include the pain associated with rheumatoid arthritis and neuromas that develop after nerve injury. The undesirable nature of opioid treatment in these patients is related not only to side-effects already mentioned and the probable duration of the disease but also to the fourth major side-effect of the opioids: dependence. Opioids such as morphine and heroin are well-known drugs of abuse that lead to physical dependence, this last side-effect involves the development of tolerance: the dose of a drug required to produce the same analgesic effect increases with time. This may lead to a condition in which the doses required to alleviate the pain are life-threatening due to the first three side-effects.

Although NSAIDs and opioids have utility in the treatment of pain there is general agreement that they are often not appropriate for the adequate treatment of pain, particularly chronic and severe pains.

Other treatments are also used, particularly for the treatment of chronic severe pain including surgical lesions of the pain pathways at several levels from

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peripheral nerves through dorsal root section and cordotomy to pituitary destruction. These are, however, mostly severe operations that are all associated with significant risk to the patient.

It can be seen, therefore, that there remains a significant need for the
5 development of new classes of pharmaceuticals for the treatment of pain of many types. The desired properties of such new therapies can be briefly expressed as follows: (1) the ability to provide significant relief of pain including severe pain; (2) the lack of systemic side effects that significantly impair the patient's quality of life; (3) long-lasting actions that do not require
10 frequent injections or long-term catheterisation of patients; (4) provision of agents that do not lead to tolerance and associated dependence.

Statement of Invention

The present invention relates to an agent which can reduce and preferably prevent the transmission of pain signals from the periphery to the central
15 nervous system, thereby alleviating the sensation of pain. Specifically, the invention can provide an agent which can reduce and preferably prevent the transmission of pain signals from nociceptive afferents to projection neurons. More specifically, the invention can provide an agent which can inhibit the exocytosis of at least one neurotransmitter or neuromodulator substance from at
20 least one category of nociceptive afferents.

In a first aspect of the invention, there is provided an agent which can be administered systemically, and can specifically target defined populations of nociceptive afferents to inhibit the release of at least one neurotransmitter or neuromodulator from the synaptic terminals of nerves.

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In a second aspect of the invention, there is provided an agent which can be locally administered at the periphery, and which is able to inhibit the release of at least one neurotransmitter or neuromodulator from the synaptic terminals of nociceptive afferents transmitting the pain signal from the periphery.

- 5 In a third aspect of the invention, an agent is provided which can be administered into the spinal cord, and which can inhibit the release of at least one neurotransmitter or neuromodulator from the synaptic terminals of nociceptive afferents terminating in that region of the spinal cord.

- 10 In a fourth aspect of the invention, there is provided an agent which can specifically target defined populations of afferent neurons, so that the effect of the agent is limited to that cell type.

In a fifth aspect of the invention, there is provided a method of treatment of pain which comprises administering an effective dose of the agent according to the invention.

- 15 In a sixth aspect of the invention, the agent can be expressed recombinantly as a fusion protein which includes the required components of the agent.

Definitions

Without wishing to be limited by the definitions set down, it is intended in this description that the following terms have the following meanings:

- 20 Light chain means the smaller of the two polypeptide chains which form clostridial neurotoxins; it has a molecular mass of approximately 50 kDa and is commonly referred to as L-chain or simply L.

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Heavy chain means the larger of the two polypeptide chains which form clostridial neurotoxins; it has a molecular mass of approximately 100 kDa and is commonly referred to as H-chain or simply H.

5 H_C fragment means a fragment derived from the H-chain of a clostridial neurotoxin approximately equivalent to the carboxy-terminal half of the H-chain, or the domain corresponding to that fragment in the intact H-chain. It contains the domain of the natural toxin involved in binding to motor neurons.

10 H_N fragment means a fragment derived from the H-chain of a clostridial neurotoxin approximately equivalent to the amino-terminal half of the H-chain, or the domain corresponding to that fragment in the intact in the H-chain. It contains a domain involved in the translocation of the L-chain across endosomal membranes.

15 LH_N means a fragment derived from a clostridial neurotoxin that contains the L-chain, or a functional fragment thereof coupled to the H_N fragment. It is commonly derived from the intact neurotoxin by proteolysis.

Targeting Moiety (TM) means any chemical structure of an agent which functionally interacts with a binding site causing a physical association between the agent and the surface of a primary sensory afferent.

20 Binding site (BS) means a structure on the surface of a cell with which exogenous molecules are able to interact in such a way as to bring about a physical association with the cell.

Primary sensory afferent is a nerve cell that can carry sensory information from the periphery towards the central nervous system.

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Primary nociceptive afferent is a nerve cell that can carry sensory information from the periphery towards the central nervous system, where that information can result in a sensation of pain.

Brief Description of the Drawings

5 Figure 1 shows a Coomassie stain of an SDS-PAGE analysis of the fractions from size-exclusion chromatography of the products of the coupling reaction between derivatized Nerve Growth Factor (NGF) and derivatized LH_N from BoNT/A.

10 Figure 2 shows a Coomassie stain of an SDS-PAGE analysis of the conjugate of NGF and LH_N under reducing and non-reducing conditions.

Figure 3 shows a Western blot of extracts from PC12 cells treated with the conjugate of NGF and LH_N, probed with an antibody that recognises the product of the proteolysis of SNAP-25 by the L-chain of BoNT/A.

15 Figure 4 shows a Western blot of extracts from rat dorsal root ganglion neurons treated with the conjugate of NGF and LH_N, probed with an antibody that recognises the product of the proteolysis of SNAP-25 by the L-chain of BoNT/A.

Detailed Description of the Invention

20 It can be seen that, an agent for reducing or preventing the transmission of pain signals from peripheral, nociceptive afferent neurons to projection neurons has many potential applications in the reduction of the sensation of pain, particularly of severe, chronic pain.

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According to the invention, there is provided an agent which can inhibit the release of at least one neurotransmitter or neuromodulator or both from the synaptic terminals of nociceptive afferents.

The agent has a number of discrete functions:

- 5 1) It binds to a surface structure (the Binding Site [BS]) which is characteristic of, and has a degree of specificity for, nociceptive afferent neurons.
- 2) It enters the neuron. The entry of molecules into a cell can occur by a process of endocytosis. Only certain cell surface BSs undergo endocytosis, and preferably the BS to which the agent binds is one of these. In one aspect of this
10 invention, the BS is present on the peripheral, sensory fibres of the nociceptive afferent neuron and, following internalization, undergoes retrograde transport to the cell body and central processes of the neuron, in such a manner that the agent is also delivered to these regions of the neuron. In another aspect of this invention, the BS to which the agent binds is present on the central processes or
15 cell body of the nociceptive afferent neuron.
- 3) The agent enters the cytosol.
- 4) The agent modifies components of the exocytotic machinery present in the synaptic terminals of the central processes of those neurons, such that the release of at least one neurotransmitter or neuromodulator from the synaptic
20 terminal is reduced or preferably prevented.

Surprisingly, an agent of the present invention can be produced by modifying a clostridial neurotoxin or fragment thereof.

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The clostridial neurotoxins are proteins with molecular masses of the order of 150 kDa. They are produced by various species of the genus *Clostridium*, most importantly *C. tetani* and several strains of *C. botulinum*. There are at present eight different classes of the neurotoxins known: tetanus toxin, and botulinum neurotoxin in its serotypes A, B, C1, D, E, F and G, and they all share similar structures and modes of action. The clostridial neurotoxins are synthesized by the bacterium as a single polypeptide that is modified post-translationally to form two polypeptide chains joined together by a disulphide bond. The two chains are termed the heavy chain (H), which has a molecular mass of approximately 100 kDa, and the light chain (L), which has a molecular mass of approximately 50 kDa. The clostridial neurotoxins bind to an acceptor site on the cell membrane of the motor neuron at the neuromuscular junction and are internalised by an endocytotic mechanism. The internalised clostridial neurotoxins possess a highly specific zinc-dependent endopeptidase activity that hydrolyses a specific peptide bond in at least one of three proteins, synaptobrevin, syntaxin or SNAP-25, which are crucial components of the neurosecretory machinery, and this activity of the clostridial toxins results in a prolonged muscular paralysis. The zinc-dependent endopeptidase activity of clostridial neurotoxins is found to reside in the L-chain. The clostridial neurotoxins are highly selective for motoneurons due to the specific nature of the acceptor site on those neurons. The specific neuromuscular junction binding activity of clostridial neurotoxins is known to reside in the carboxy-terminal portion of the heavy chain component of the dichain neurotoxin molecule, a region known as H_C.

Surprisingly, by covalently linking a clostridial neurotoxin, or a hybrid of two clostridial neurotoxins, in which the H_C region of the H-chain has been removed or modified, to a new molecule or moiety, the Targeting Moiety (TM), that binds to a BS on the surface of sensory neurons, a novel agent capable of inhibiting the release of at least one neurotransmitter or neuromodulator from

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nociceptive afferents is produced. A further surprising aspect of the present invention is that if the L-chain of a clostridial neurotoxin, or a fragment of the L-chain containing the endopeptidase activity, is covalently linked to a TM which can also effect internalisation of the L-chain, or fragment thereof, into the cytoplasm of a sensory neuron, this also produces a novel agent capable of inhibiting the release of at least one neurotransmitter or neuromodulator. The covalent linkages used to couple the component parts of the agent may include appropriate spacer regions.

The TM provides specificity for the BS on the nociceptive afferent neuron. The TM component of the agent can comprise one of many cell binding molecules, including, but not limited to, antibodies, monoclonal antibodies, antibody fragments (Fab, F(ab)₂, Fv, ScFv, etc.), lectins and ligands to the receptors for hormones, cytokines, growth factors or neuropeptides. A list of possible TMs is given in Table 1, this list is illustrative and is not intended to be limiting to the scope of TMs which could fulfil the requirements of this invention. In one embodiment of the invention the TM binds to a BS which undergoes retrograde transport.

It is known in the art that the H_C portion of the neurotoxin molecule can be removed from the other portion of the heavy chain, known as H_N, such that the H_N fragment remains disulphide linked to the light chain (L-chain) of the neurotoxin molecule to provide a fragment known as LH_N. Thus, in one embodiment of the present invention the LH_N fragment of a clostridial neurotoxin is covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the H_C domain of a clostridial neurotoxin is mutated or modified, e.g. by chemical modification, to reduce or preferably incapacitate its ability to bind the neurotoxin to receptors at the

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neuromuscular junction. This modified clostridial neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

5 In another embodiment of the invention, the heavy chain of a clostridial neurotoxin, in which the H_C domain is mutated or modified, e.g. by chemical modification, to reduce or preferably incapacitate its ability to bind the neurotoxin to receptors at the neuromuscular junction is combined with the L-chain of a different clostridial neurotoxin. The hybrid, modified clostridial neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

10 In another embodiment of the invention, the H_N portion of a clostridial neurotoxin is combined with the L-chain of a different clostridial neurotoxin. The hybrid LH_N is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

15 In another embodiment of the invention the light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, is linked, using linkages which may include one or more spacer regions, to a TM which can also effect the internalization of the light chain, or fragment thereof containing endopeptidase activity, into the cytoplasm of the cell.

20 In another embodiment of the invention the agent is expressed recombinantly as a fusion protein which includes an appropriate fragment of a Targeting Moiety in addition to any desired spacer domains. The recombinantly expressed agent may be derived wholly from the gene encoding one serotype of neurotoxin or be a chimaera derived from the genes encoding two different serotypes.

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In another embodiment of the invention the required LH_N , which may be a hybrid of an L and H_N from different clostridial toxin types, is expressed recombinantly as a fusion protein with the TM, and may also include one or more spacer regions.

5 In another embodiment of the invention the light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, is expressed recombinantly as a fusion protein with a TM which can also affect the internalization of the light chain, or fragment thereof containing the endopeptidase activity, into the cytoplasm of the cell. The expressed fusion
10 protein may also include one or more spacer regions.

The basis of this disclosure is the creation of novel agents with very specific and defined activities against a limited and defined class of neurons (primary sensory afferents), and as such the agents may be considered to represent a form of neurotoxin. The therapeutic use of native botulinum neurotoxins is well
15 known in the prior art. The mode of action of the botulinum neurotoxins, as described in the prior art, however, is by a mechanism, inhibition of acetylcholine secretion, and against a category of target neurons, efferent motoneurons, clearly distinct from the agents described in this disclosure. The prior art does not teach either the activity or the chemical structure of the agents
20 disclosed. Thus, although, as discussed in this application, the prior art teaches much about the native clostridial neurotoxins, native unmodified clostridial neurotoxins are not the subject of this disclosure. The agent of this invention requires modification of the clostridial neurotoxins such that the targeting property taught in the prior art is removed. The modified neurotoxin is then
25 coupled to a new targeting function (the TM), to give a novel agent with new biological properties distinct from those of the native clostridial neurotoxins and not taught in the prior art. It is this new agent with novel properties that is the subject of this disclosure.

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Exploitation in Industry

The agent described in this invention can be used in vivo, either directly or as a pharmaceutically acceptable salt, for treatment of pain.

For example, an agent according to the invention can be used systemically for the treatment of severe chronic pain. A specific example of this is the use in treatment of clinical pain associated with rheumatoid arthritis affecting multiple joints.

In another example, an agent according to the invention can be locally applied for the treatment of pain. A specific example of this is treatment by local injection into a joint affected by inflammatory pain.

In further example an agent according to the invention can be administered by spinal injection (epidural or intrathecal) at the level of the spinal segment involved in the innervation of an affected organ for the treatment of pain. This is, for example, applicable in the treatment of deep tissue pain, such as chronic malignant pain.

The present invention will now be illustrated by reference to the following non-limiting examples:

Example 1. Synthesis of a conjugate of NGF and the LH_n fragment of BoNT/A.

Lyophilised murine 2.5 S NGF was dissolved by the addition of water and dialysed into MES buffer (0.1 M MES, 0.1 M sodium chloride, pH 5.0). To this solution (at a concentration of about 0.3 mg/ml) was added PDPH (100 mg/ml in DMF) to a final concentration of 1 mg/ml. After mixing, solid EDAC was

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added to produce a final concentration of about 0.2 mg/ml. The reaction was allowed to proceed for at least 30 min at room temperature. Excess PDPH was then removed by desalting over a PD-10 column (Pharmacia) previously equilibrated with MES buffer.

- 5 The LH_N fragment of BoNT/A was produced essentially by the method of Shone C.C., Hambleton, P., and Melling, J. 1987, *Eur. J. Biochem.* 167, 175-180. An amount of LH_N equivalent to half the weight of NGF used dissolved in triethanolamine buffer (0.02 M triethanolamine/HCl, 0.1 M sodium chloride, pH 7.8) at a concentration of about 1 mg/ml, was reacted with Traut's reagent (100 mM stock solution in 1 M triethanolamine/HCl, pH 8.0) at a final concentration of 2 mM. After one hour the LH_N was desalted into PBSE (phosphate buffered saline with 1 mM EDTA) using a PD-10 column (Pharmacia). The protein peak from the column eluate was concentrated using a Microcon 50 (Amicon) to a concentration of about 2 mg/ml.
- 10
- 15 The derivatized NGF was subjected to a final concentration step resulting in a reduction in volume to less than 10 % of the starting volume and then mixed with the derivatized LH_N overnight at room temperature. The products of the reaction were analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl-sulphate (SDS-PAGE).
- 20 The conjugate resulting from the above reaction was partially purified by size exclusion chromatography over Bio-Gel P-100 (Bio-Rad). The elution profile was followed by measuring the optical density at 280 nm and SDS-PAGE analysis of the fractions. This allowed the separation of conjugate from free NGF and by-products of the reaction.
- 25 Fig. 1 shows the SDS-PAGE analysis of the fractions from one such Bio-Gel P-100 column. The free LH_N and conjugate (M, 100kDa and above) are clearly

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separated from the majority of the free NGF (M_r 13kDa). As 2.5S NGF is a homo-dimer formed by non-covalent interactions it is dissociated by treatment with SDS. Thus molecules that have formed covalent cross-links to LH_N through one subunit only will dissociate during the SDS-PAGE analysis and give rise to the free NGF band seen in fractions 4-6. This result demonstrates that the homo-dimeric structure of NGF remains intact after derivatisation. The free LH_N seen in these fractions represents a minor component which has not coupled to NGF. Fractions 4-6 were pooled before further analysis.

Fig. 2 shows an analysis of the conjugate by SDS-PAGE under reducing and non-reducing conditions. Lane 1 is free LH_N under non-reducing conditions, lane 2 is the same amount of LH_N reduced with 50 mM dithiothreitol. Lanes 3 and 4 show the conjugate after size exclusion chromatography either without (lane 3) or with (lane 4) reduction by dithiothreitol. Similarly, lanes 5 and 6 show NGF without or with reduction respectively. The results clearly show that the material in lane 5 with an apparent molecular mass greater than 100kDa produces, upon reduction, the constituent bands of LH_N and NGF only. Furthermore the intensity of the bands following reduction is such that they must be derived from material other than the small amounts of free LH_N and NGF observed in the unreduced sample. The only available source for the excess is the material with an apparent molecular mass >100kDa. The conjugate in the fractions obtained following the size-exclusion chromatography thus represents NGF and LH_N covalently linked by reducible disulphide linkages.

The fractions containing conjugate were stored at 4°C until required.

Example 2. Activities of a conjugate of NGF and LH_N in PC-12 cells.

PC12 cells are a cell-line of neuroectodermal derivation that are commonly used as a model system for the study of nerve function. As a model system for testing the function of a conjugate of NGF and LH_N they have two necessary features: firstly they are well known to possess cell-surface receptors for NGF that have been shown to be involved in a differentiation process in response to low concentrations of NGF. Secondly they have been shown to contain the exocytotic machinery for neurotransmitter release including, importantly in this example, SNAP-25.

PC12 cells were plated out into a 24-well plate that had been coated with MATRIGEL basement membrane matrix (Collaborative Biomedical Products) at a density of approximately 5×10^5 cells per well. After a few days in culture (RPMI 1640 with 2 mM glutamine, 10% horse serum and 5% foetal calf serum, 37°C, 5% CO₂) the medium was replaced with fresh medium containing added conjugate (prepared as described in Example 1) or LH_N or no addition. After being kept in culture overnight the medium was removed and the cells washed once with fresh medium. Cells were then lysed by the addition of 0.45 ml sodium hydroxide (0.2 M) for 30 min. After this time the solutions were neutralised by the addition of 0.45 ml hydrochloric acid (0.2 M) followed by 0.1 ml of HEPES/NaOH (1 M, pH 7.4). To extract the membrane proteins from these mixtures Triton-X-114 (10%, v/v) was added and incubated at 4°C for 60 min, the insoluble material was removed by centrifugation and the supernatants were then warmed to 37°C for 30 min. The resulting two phases were separated by centrifugation and the upper phase discarded. The proteins in the lower phase were precipitated with chloroform/methanol for analysis by Western blotting.

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The samples were separated by SDS-PAGE and transferred to nitro-cellulose. Proteolysis of SNAP-25, a crucial component of the neurosecretory process and the substrate for the zinc-dependent endopeptidase activity of BoNT/A, was then detected by probing with an antibody that recognises the newly revealed
5 carboxy-terminus of the cleaved SNAP-25 (the antibody is described in Patent Application PCT/GB95/01279). Figure 3 shows an example of such a Western blot. No significant immunoreactivity was observed in samples from control cells (lanes 1 and 2) whereas a band corresponding to a molecular mass of 29 kDa was observed weakly in samples incubated with 10 mg/ml LH_N (lanes 5
10 and 6) and strongly in samples incubated with 10 mg/ml of the conjugate of NGF and LH_N (lanes 3 and 4). Thus incubation of PC12 cells with the conjugate leads to the marked proteolysis of SNAP-25 indicating that the conjugate has introduced the zinc-dependent proteolytic activity of the L-chain of BoNT/A into the cells' cytoplasm. Little or no such activity was seen with
15 the constituent components of the conjugate.

Incubation of cells with the conjugate in the presence of an excess of free NGF resulted in a reduced production of the proteolytic product of SNAP-25 than did incubation with the conjugate alone. This indicates that the action of the conjugate occurs by means of the NGF targeting moiety interacting with the cell
20 surface receptors for NGF.

Example 3. The activity of a conjugate of NGF and LH_N in primary cultures of dorsal root ganglion neurons.

The dorsal root ganglia contain the cell bodies of primary nociceptive afferents. It is well established that in primary *in vitro* cultures of this tissue the neurons
25 retain many of the characteristics of the nociceptive afferents. These characteristics include the ability to release neuropeptides such as substance P in

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response to chemical stimuli known to cause pain *in vivo* (e.g. capsaicin).

Furthermore the neurons are known to possess receptors for NGF.

Primary cultures of dorsal root ganglion neurons were established following dissociation of the ganglia dissected from rat embryos (embryological age 12-15
5 days). The cells were plated into 12 well plates at an initial density of 3×10^5 cells/well in a medium containing NGF (100 ng/ml). After one day in culture fresh medium containing cytosine arabinoside (10 mM) was added to kill non-neuronal cells. The cytosine arabinoside was removed after 2-4 days. After several more days in culture the medium was replaced with fresh medium
10 containing conjugate or LH_N in the absence of NGF. Following overnight incubation at 37°C the medium was removed, the cells were lysed and the hydrophobic proteins extracted using Triton-X-114 as described in Example 2.

The samples were analysed by Western blotting as described in Example 2 with the antibody that recognises the product of the BoNT/A proteolysis of SNAP-
15 25. No immunoreactivity was observed in samples from control cells (lane 4) whereas a band corresponding to a molecular mass of 29kDa was observed weakly in samples incubated with 10 mg/ml LH_N (lane 3) and strongly in samples incubated with 10 mg/ml of the conjugate of NGF and LH_N (lanes 1 and 2).

20 This result indicates that the conjugate can deliver the proteolytically-active L-chain of BoNT/A into the cytoplasm of the neuronal cells that, *in vivo*, form the primary nociceptive afferents.

Example 4. The production of a chimeric LH_N whereof the L chain is derived from BoNT/B and the H_N fragment from BoNT/A.

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The H_N fragment of BoNT/A is produced according to the method described by Shone C.C., Hambleton, P., and Melling, J. (1987, *Eur. J. Biochem.* 167, 175-180) and the L-chain of BoNT/B according to the method of Sathyamoorthy, V. and DasGupta, B.R. (1985, *J. Biol. Chem.* 260, 10461-10466). The free
5 cysteine on the H_N fragment of BoNT/A is then derivatised by the addition of a ten-fold molar excess of dipyridyl disulphide followed by incubation at 4°C overnight. The excess dipyridyl disulphide and the thiopyridone by product are then removed by desalting the protein over a PD10 column (Pharmacia) into PBS.

10 The derivatised H_N is then concentrated to a protein concentration in excess of 1 mg/ml before being mixed with an equimolar portion of L-chain from BoNT/B (>1 mg/ml in PBS). After overnight incubation at room temperature the mixture is separated by size exclusion chromatography over Superose 6 (Pharmacia), and the fractions analysed by SDS-PAGE. The chimeric LH_N is
15 then available for derivatisation to produce a targeted conjugate as described in Example 1.

The examples described above are purely illustrative of the invention. In synthesizing the agent the coupling of the TM to the modified clostridial neurotoxin or fragment thereof is achieved via chemical coupling using reagents
20 and techniques known to those skilled in the art. Thus, although the examples given use exclusively the PDPH/EDAC and Traut's reagent chemistry any other coupling chemistry capable of covalently attaching the TM component of the agent to clostridial neurotoxin derived component and known to those skilled in the art is covered by the scope of this application. Similarly it is evident to
25 those skilled in the art that either the DNA coding for either the entire agent or fragments of the agent could be readily constructed and, when expressed in an appropriate organism, could be used to recombinantly produce the agent or fragments of the agent. Such genetic constructs of the agent of the invention

obtained by techniques known to those skilled in the art are also covered in the scope of this invention.

Table 1- Possible Targeting Moieties (TM)**Growth Factors:**

1. Nerve growth factor (NGF);
2. Leukaemia inhibitory factor (LIF);
- 5 3. Basic fibroblast growth factor (bFGF);
4. Brain-derived neurotrophic factor (BDNF);
5. Neurotrophin-3 (NT-3);
6. Hydra head activator peptide (HHAP);
7. Transforming growth factor 1 (TGF- 1);
- 10 8. Transforming growth factor 2 (TGF- 2);
9. Transforming growth factor (TGF-);
10. Epidermal growth factor (EGF);
11. Ciliary neuro-trophic factor (CNTF).

Cytokines:

- 15 1. Tumour necrosis factor (TNF-);

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2. Interleukin-1 (IL-1);
3. Interleukin-1 (IL-1);
4. Interleukin-8 (IL-8).

Peptides:

- 5 1. -Endorphin;
2. Methionine-enkephalin;
3. D-Ala²-D-Leu⁵-enkephalin;
4. Bradykinin.

Antibodies:

- 10 1. Antibodies against the lactoseries carbohydrate epitopes found on the surface of dorsal root ganglion neurons (e.g. monoclonal antibodies 1B2 and LA4);
2. Antibodies against any of the receptors for the ligands given above.
3. Antibodies against the surface expressed antigen Thy1 (e.g. monoclonal
15 antibody MRC OX7).

Claims

1. An agent exhibiting specificity for peripheral sensory afferents which comprises a molecule capable of introducing a protease activity specific for components of the neurosecretory machinery into the cytosol of a primary
5 sensory afferent, and thereby inhibiting the transmission of signals between a primary sensory afferent and a projection neuron by controlling the release of at least one neurotransmitter or neuromodulator from the primary sensory afferent.
2. An agent according to Claim 1 in which the protease activity is the protease activity of the light chain (L-chain) of a clostridial neurotoxin or a
10 fragment thereof retaining the protease activity.
3. An agent according to any preceding Claim which comprises a Targeting Moiety (TM) coupled to a clostridial neurotoxin in which the heavy chain (H-chain) is removed or modified, the TM being capable of functionally interacting with a binding site causing a physical association between the agent and the
15 surface of a primary sensory afferent.
4. An agent according to any preceding Claim which comprises a Targeting Moiety (TM) coupled to a clostridial neurotoxin in which the H_C part of the H-chain is removed or modified.
5. An agent according to Claim 4 in which the modified H-chain is the H_N-
20 fragment of a clostridial neurotoxin.
6. An agent according to any of Claims 2-5 in which the clostridial neurotoxin component is obtained from botulinum neurotoxin.

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7. An agent according to any of Claims 2-6 in which the clostridial neurotoxin component is obtained from botulinum neurotoxin type A.
8. An agent according to any of Claims 2-6 in which the clostridial neurotoxin component is obtained from botulinum neurotoxin type B.
- 5 9. An agent according to any of Claims 2-6 in which the clostridial neurotoxin component is obtained from botulinum neurotoxin type C₁.
- 10 10. An agent according to any of Claims 2-9 in which the H-chain is modified by chemical derivatisation to reduce or remove its native binding affinity for motor neurons.
11. An agent according to any of Claims 2-9 in which the H-chain is modified by mutation to reduce or remove its native binding affinity for motor neurons.
12. An agent according to any of Claims 2-9 in which the H-chain is modified by proteolysis to reduce or remove its native binding affinity for motor neurons.
- 15 13. An agent according to Claim 12 which is formed by the coupling of a TM to the LH_N fragment of botulinum neurotoxin type A.
14. An agent according to Claim 12 which is formed by the coupling of a TM to the LH_N fragment of botulinum neurotoxin type B.
- 20 15. An agent according to Claim 12 which is formed by the coupling of a TM to the LH_N fragment of botulinum neurotoxin type C₁.

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16. An agent according to any of Claims 2-12 in which the H-chain is obtained from a different clostridial neurotoxin than that from which the L-chain is obtained.
17. An agent according to Claim 16 in which the H-chain is obtained from botulinum neurotoxin type A and the L-chain from botulinum neurotoxin type B.
18. An agent according to Claim 17 which is composed of a TM linked to the H_N fragment of botulinum neurotoxin type A and the L-chain of botulinum neurotoxin type B.
19. An agent according to any of Claims 2-18 in which the L-chain or L-chain fragment is linked to the H-chain by a direct covalent linkage.
20. An agent according to any of Claims 2-18 in which the L-chain or L-chain fragment is linked to the H-chain by a covalent linkage which includes one or more spacer regions.
21. An agent according to any of Claims 2-20 in which the TM is capable of delivering the L-chain or L-chain fragment into the cytosol of a primary sensory afferent unaided.
22. An agent according to any of Claims 2-21 in which the ability to deliver the L-chain or L-chain fragment into the cytosol of a primary sensory afferent is entirely contained within the TM.
23. An agent according to any of Claims 3-22 in which the TM comprises a ligand to a cell-surface binding site on a primary sensory afferent.

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24. An agent according to any of Claims 3-23 in which the TM binds to a binding site which is characteristic of a particular defined population of primary sensory afferents.
25. An agent according to any of Claims 3-24 in which the TM binds to a binding site which is characteristic of a particular defined population of primary nociceptive afferents.
26. An agent according to any of Claims 3-25 in which the TM binds to a binding site which undergoes retrograde transport within a primary sensory afferent.
27. An agent according to any of Claims 3-26 in which the TM binds to a binding site which undergoes retrograde transport within a primary nociceptive afferent.
28. An agent according to any of Claims 3-27 in which the TM comprises a ligand to a cell-surface receptor on a primary sensory afferent.
29. An agent according to any of Claims 3-28 in which the TM comprises a ligand to a growth factor receptor on a primary sensory afferent.
30. An agent according to any of Claims 3-28 in which the TM comprises a ligand to a neuropeptide receptor on a primary sensory afferent.
31. An agent according to any of Claims 3-28 in which the TM comprises a ligand to a cytokine receptor on a primary sensory afferent.
32. An agent according to any of Claims 3-28 in which the TM comprises a ligand to a hormone receptor on a primary sensory afferent.

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33. An agent according to any of Claim 3-32 in which the TM comprises a monoclonal antibody or is derived from a monoclonal antibody to a surface antigen on a primary sensory afferent.
34. An agent according to Claim 29 in which the TM comprises a ligand to a nerve growth factor receptor.
35. An agent according to Claim 34 in which the TM comprises nerve growth factor.
36. An agent according to Claim 35 which comprises nerve growth factor linked to the LH_N fragment of botulinum neurotoxin type A.
37. An agent according to any preceding Claim in which the TM is linked to the clostridial neurotoxin-derived component by a direct covalent linkage.
38. An agent according to any preceding Claim in which the TM is linked to the clostridial neurotoxin-derived component by a covalent linkage which includes one or more spacer regions.
39. An agent according to any preceding Claim which prevents the release of a neurotransmitter or neuromodulator from a primary sensory afferent.
40. An agent according to any preceding Claim which inhibits the release of a neurotransmitter or neuromodulator from a primary nociceptive afferent.
41. A method for obtaining an agent according to any preceding Claim which comprises the covalent attachment of a TM to a modified clostridial neurotoxin or a fragment of a clostridial neurotoxin.

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42. A method for obtaining an agent according to any of Claims 1-40 which comprises the covalent attachment of a TM to a modified clostridial neurotoxin or a fragment of a clostridial neurotoxin with the inclusion of one or more spacer regions.

5 43. A method for obtaining an agent according to any of Claims 1-40 which comprises constructing a genetic construct which codes for a modified clostridial neurotoxin or a fragment of a clostridial neurotoxin, incorporating said construct into a host organism, expressing the construct to produce the modified clostridial neurotoxin or fragment of a clostridial neurotoxin and the
10 covalent attachment of the modified clostridial neurotoxin or fragment of a clostridial neurotoxin to a TM.

44. A method for obtaining an agent according to any of Claims 1-40 which comprises constructing a genetic construct which codes for a modified clostridial neurotoxin or a fragment of a clostridial neurotoxin, incorporating
15 said construct into a host organism, expressing the construct to produce the modified clostridial neurotoxin or fragment of a clostridial neurotoxin and the covalent attachment of the modified clostridial neurotoxin or fragment of a clostridial neurotoxin to a TM with the inclusion of one or more spacer regions.

45. A method for obtaining an agent according to any of Claims 1-40 which
20 comprises constructing a genetic construct which codes for the agent, incorporating said construct into a host organism and expressing the construct to produce the agent.

46. A method of controlling the release of a neurotransmitter or neuromodulator from a primary sensory afferent by applying the agent of any
25 one of Claims 1-40.

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47. A method of controlling the release of a neurotransmitter or neuromodulator from a primary nociceptive afferent by applying the agent of any one of Claims 1-40.
- 5 48. A method of controlling the transmission of sensory information from a primary sensory afferent to a projection neuron by applying the agent of any one of Claims 1-40.
49. A method of controlling the transmission of sensory information from a primary nociceptive afferent to a projection neuron by applying the agent of any one of Claims 1-40.
- 10 50. A method of controlling the sensation of pain by applying the agent of any one of Claims 1-40.
51. Use of the agent according to any one of Claims 1-40 or a pharmaceutically acceptable salt thereof as a medicament for the alleviation of pain.
- 15 52. Use of the agent according to any one of Claims 1-40 or a pharmaceutically acceptable salt thereof as a medicament for the prevention of pain.
53. Use of the agent according to any one of Claims 1-40 in the manufacture of a medicament for the alleviation of pain.
- 20 54. Use of the agent according to any one of Claims 1-40 in the manufacture of a medicament for the prevention of pain.

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55. A method of alleviating pain which comprises administering an effective dose of the agent according to any one of Claims 1-40.

56. A method of preventing pain which comprises administering an effective dose of the agent according to any one of Claims 1-40.

5 57. An agent exhibiting specificity for peripheral sensory afferents which can inhibit the release of at least one neurotransmitter or neuromodulator or both from the synaptic terminals of nociceptive afferents, the agent having the following discreet functions:

- 10 1) It binds to a surface structure (the Binding Site [BS]) which is characteristic of, and has a degree of specificity for, nociceptive afferent neurons;
- 2) It enters the neuron;
- 3) It enters the cytosol; and
- 15 4) It modifies components of the exocytotic machinery present in the synaptic terminals of the central processes of the neurons, such that the release of at least one neurotransmitter or neuromodulator from the synaptic terminal is reduced or prevented.

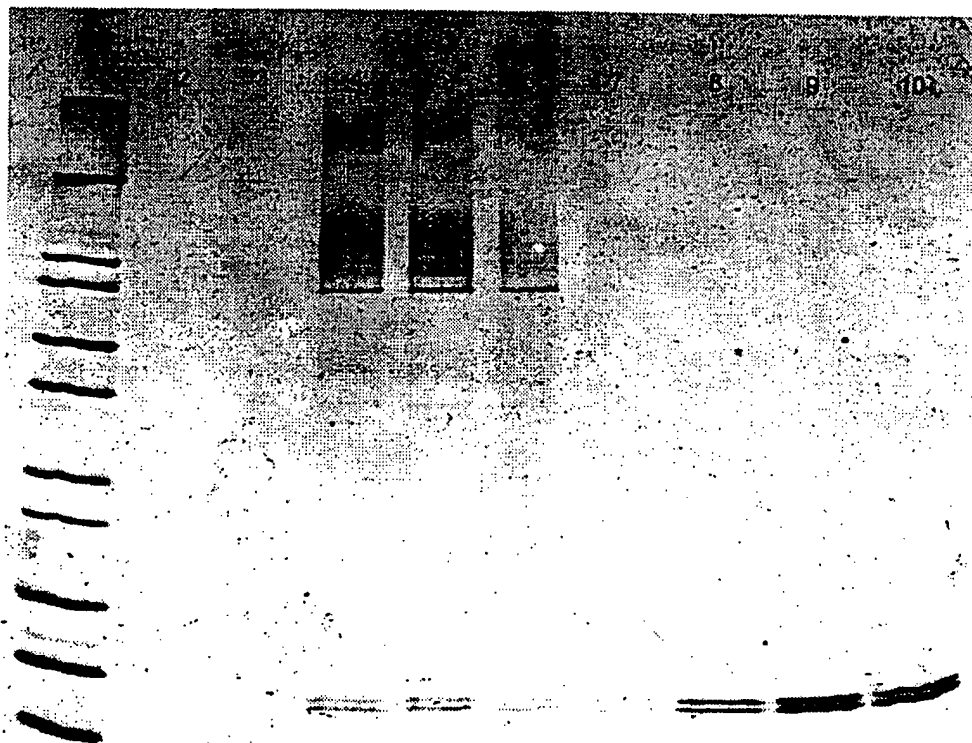


Figure 1

2/4

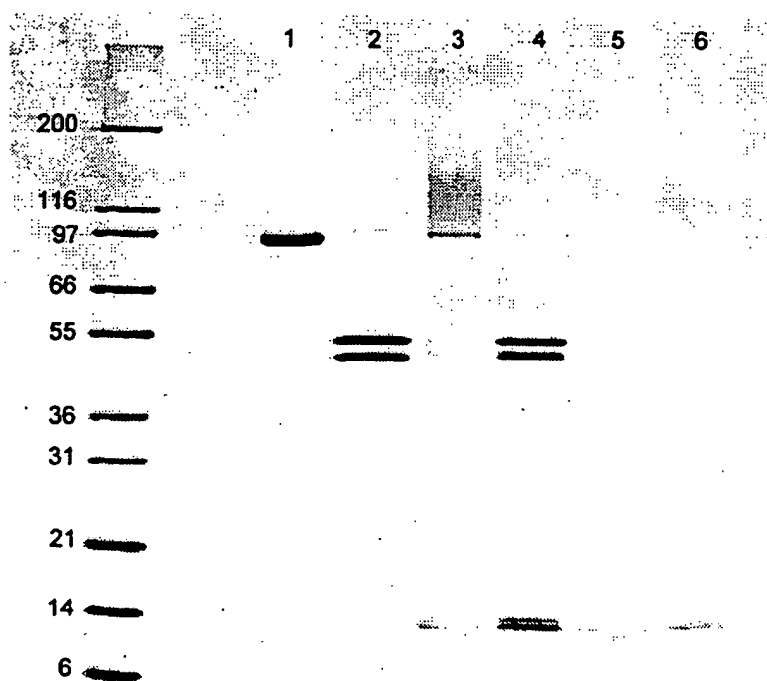


Figure 2

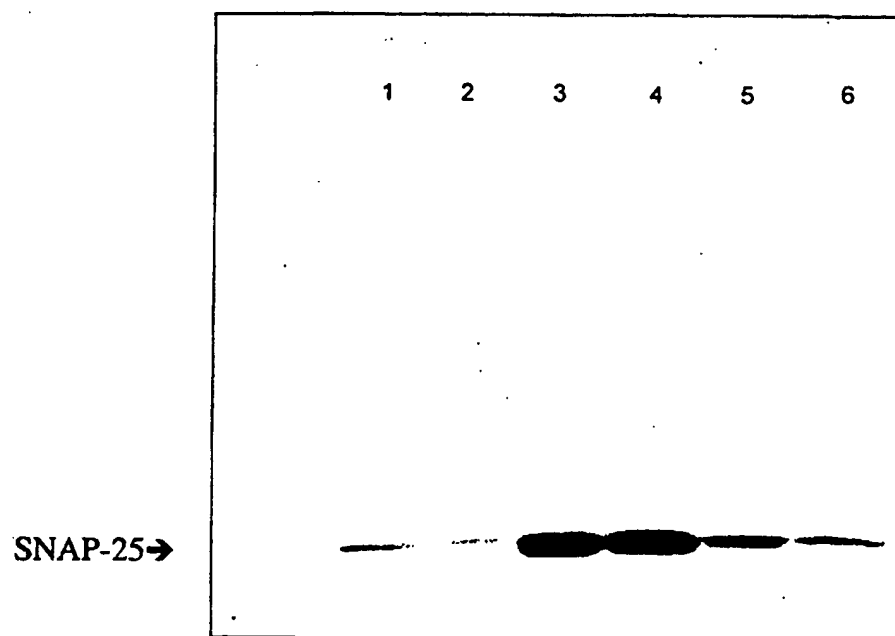


Figure 3

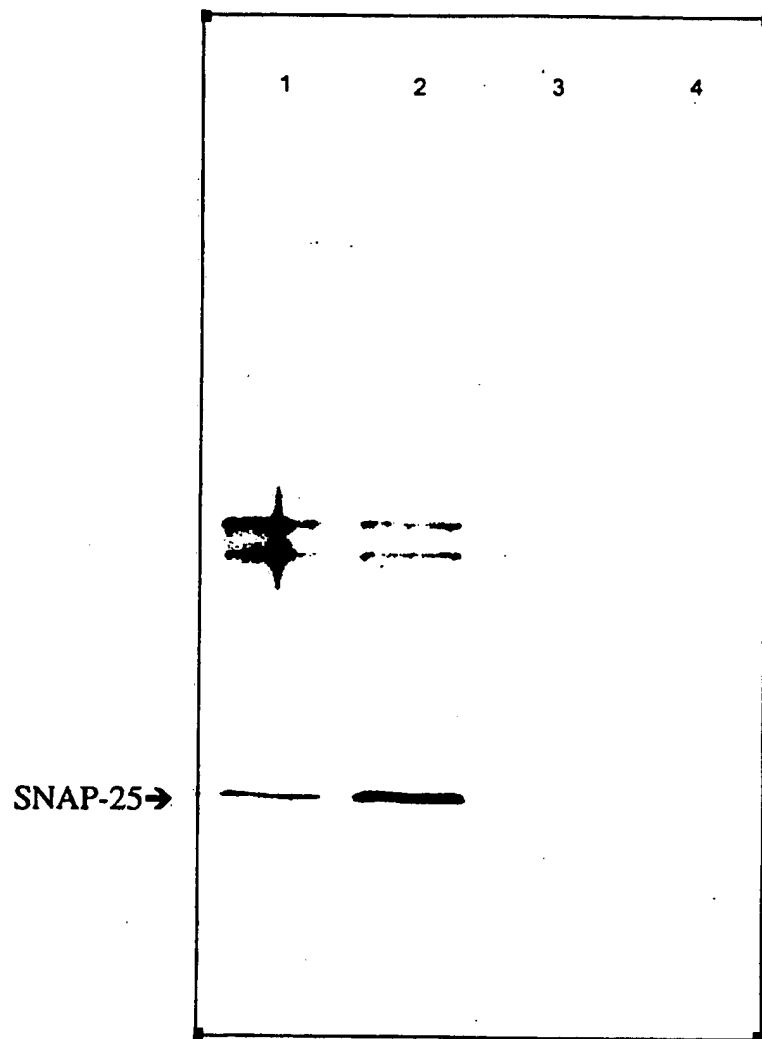


Figure 4

INTERNATIONAL SEARCH REPORT

International Application No
PCT, GB 96/00916

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/31 C07K19/00 C07K14/33 C12N9/52 C07K14/48
C07K14/475 C07K14/52 C07K14/575 C07K16/18 C12N15/62
A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,94 15629 (ASS SYNAPSE BIOLOG) 21 July 1994 see the whole document ---	1-15, 19-57
Y	WO,A,94 28923 (ALLERGAN INC) 22 December 1994 see the whole document ---	1-15, 19-57
Y	JOURNAL OF NEUROLOGY, vol. 239, 1992, pages 16-20, XP002009346 P. HAMBLETON: "Clostridium botulinum toxins: a general review of involvement in disease, structure, and mode of action and preparation for clinical use" see the whole document --- -/-	1-15, 19-57

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *&* document member of the same patent family

Date of the actual completion of the international search

25 July 1996

Date of mailing of the international search report

01.08.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Van der Schaal, C

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/00916

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 129 434 (UNIV TEXAS) 27 December 1984 see the whole document ---	1-15, 19-57
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, no. 33, 25 November 1992, MD US, pages 23479-23483, XP002009347 G. SCHIAVO ET AL: "Botulinum neurotoxins are zinc proteins" see page 23479, left-hand column, last line - right-hand column; figure 1 ---	2-15, 19-56
Y	MEDLINE DATABASE Accession Nr. 9563023 XP002009349 & HOKKAIDO JOURNAL OF MEDICAL SCIENCE, vol. 70, no. 1, January 1995, pages 19-28, N. FUJII: "Structure and function of botulinum toxin" see abstract ---	2-15, 19-56
Y	MEDLINE DATABASE Accession Nr. 96018139 XP002009350 & TOXICON, vol. 33, no. 4, April 1995, pages 559-567, F. LEBEDA AND M. OLSON: "Structural predictions of the channel-forming region of botulinum heavy chain" see abstract ---	2-15, 19-56
Y	BIOLOGICAL ABSTRACTS, vol. 97, Philadelphia, PA, US; abstract no. 225145, POULAIN B: "Molecular mechanism of action of tetanus toxin and botulinum neurotoxins." XP002009351 see abstract & PATHOLOGIE BIOLOGIE 42 (2). 1994. 173-182. ISSN: 0369-8114, ---	2-15, 19-56
Y	WO,A,95 01806 (KONDRATYEV ALEXEI) 19 January 1995 see the whole document ---	21-32, 34-56
Y	EP,A,0 544 292 (BOEHRINGER MANNHEIM GMBH) 2 June 1993 see the whole document ---	21-32, 34-56

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 96/00916

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 265, no. 16, 1990, MD US, pages 9153-9158, XP002009348 T. BINZ ET AL: "The complete sequence of botulinum neurotoxin type A..." see the whole document -----</p>	43-45

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB96/00916

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 46-52, 55 and 56
because they relate to subject matter not required to be searched by this Authority, namely:
Although these claims are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.